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TITLE: Targeting Palmitoyl Acyltransferases in Mutant NRAS-Driven Melanoma

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14. ABSTRACT Mutations in N-RAS are linked to ~20% of melanoma with no effective treatment. Targeting palmitoyl acyltransferases (PATs) involved in N-RAS regulation could be a novel strategy to treat N-RAS mutant melanoma. The objective of the project is to identify PATs responsible for NRAS activation in melanoma cells using chemical biology and functional genomic approaches. In the first year of the study, we have developed more potent chemical probes to profile PATs in cells, and have carried out PATs profiling in melanoma cells using chemical probes and mRNA profiling. We have identified candidate PATs highly expressed in NRAS melanoma cells. We have developed shRNA reagents to further study their functions.					
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## 1. INTRODUCTION

Mutations in N-RAS are linked to ~20% of melanoma with no effective treatment, representing a large unmet medical need. Palmitoylation (post-translational modification by adding a 16-carbon palmitate) is required for N-RAS oncogenic activity. Recently, 23 of DHHC (Asp-His-His-Cys)-family proteins were discovered as protein palmitoyl acyltransferases (PATs). Targeting DHHC-PATs involved in N-RAS regulation could be a novel strategy to treat N-RAS mutant melanoma. We have developed chemical probes that covalently label the active sites of DHHC-PATs, allowing us to investigate the enzymatic activities of PATs that are responsible for N-RAS palmitoylation using Activity-Based Protein Profiling (ABPP) approaches. The first objective is to identify DHHC-PATs regulating palmitoylation of oncogenic N-RAS in melanoma cells using ABPP methods and compare with the mRNA expression profile to identify candidate PATs highly active in NRAS mutant melanoma cells. The second objective is to evaluate the effects of shRNAs targeting DHHC-PATs on N-RAS activity, melanoma cell proliferation and apoptosis. Finally, we aim to design and synthesize analogues of cerulenin, a natural product inhibitor of PATs to identify small molecule inhibitors of N-RAS palmitoylation.

## 2. KEYWORDDD

NRAS, melanoma, palmitoylation, DHHC domain containing palmitoyl acyltransferases, Activity based protein profiling (ABPP), proteomics

## 3. ACCOMPLISHMENTS

### What were the major goals of the project?

The major goal of the project is to use chemical biology and functional genomics approaches to study palmitoyl acyltransferases involved in NRAS activation in melanoma cells. As we stated in the SOW of the grant, there are three major tasks of this project. Task1: To identify DHHC-PATs regulating palmitoylation of oncogenic N-RAS in melanoma cells (Month 1-12). Task2. To evaluate the effects of shRNAs targeting DHHC-PATs on NRAS activity, melanoma cell proliferation and apoptosis (Month 13-24). Task3. To design and synthesize analogues of cerulenin, a natural product inhibitor of palmitoyl acyltransferases to identify small molecule inhibitors of N-RAS palmitoylation. (in parallel with Task1 and 2, Month 0-24).

In the last funding period, as we stated in the SOW, our work is focused on Task 1 and we have initiated some work in Task 3. Below are the details of our activities regarding to the SOW.

### What was accomplished under these goals?

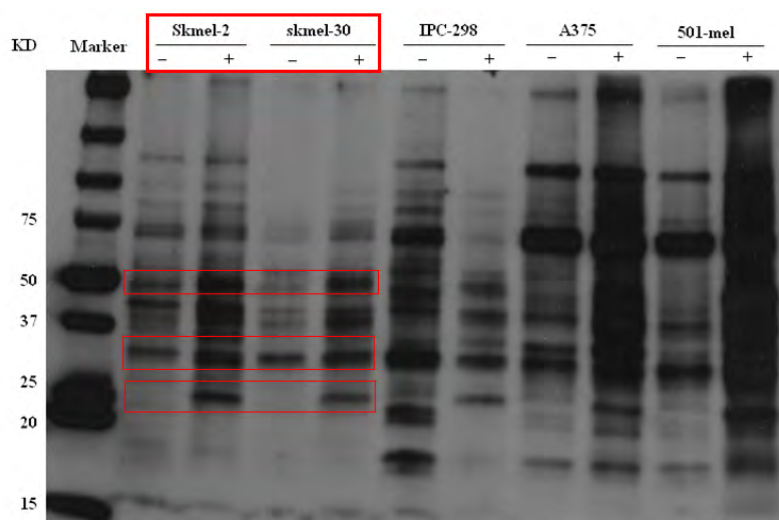
**Task 1a.** Test the activity based probe (16C-BYA) for labeling efficiency in multiple N-RAS mutant melanoma cell lines (SK-Mel30, SK-Mel2, MEL-JUSO, BL, etc.) and control cell lines (BRAF mutant melanoma, A375, U62, MalMe, M14, SK-Mel5 and primary

human melanocytes) in 6-well format, optimize the concentration and labeling time (month 1-6).

**Accomplishments:** We have tested the labeling of the probe 16C-BYA (alkyne-2-BP) in NRAS melanoma cells (SK-Mel30, SK-Mel2 and 501Mel), and compared the pattern of labeling with the control cell line A375, which carries BRAF mutation. The concentration and time of labeling has been optimized to 50uM of probe for 8 hours labeling. We have carried out Click chemistry using Biotin-Azide or Rhodamine-Azide, and have compared the labeling efficiency and pattern of the cell lines that we have studied. We found that the probe specifically labeled several bands in NRAS mutant cell lines, which might represent potential candidate palmitoyl acyltransferases regulating NRAS activities (Figure 1).

**Task 1b.** Proteomic and mass spectrometry analysis of labeled proteins (month 6-12).

**Accomplishments:** We have carried out large-scale labeling experiments under the optimized condition (100uM probe for 8hours labeling) using 5 dishes of 10cm dishes for SK-Mel2 and SK-Mel30 cells, which gave us sufficient proteins for follow-up proteomics studies. We carried out Click chemistry using Biotin-azide, and used Streptavidin bead to enrich the labeled protein. We carried out on-bead digestion using trypsin and the peptides were submitted to Harvard Medical School Taplin Mass Spectrometry center for protein identification. We successfully finished the proteomics studies, and our studies suggested that 3 DHHC proteins were among the palmitoyl acyltransferases with high activities in the NRAS cell lines. These protein will be candidate PATs for further studies. We also carried out qRT-PCR studies of the NRAS mutant cell line (SK-Mel30) and BRAF mutant cell line (A375) of all 23 DHHC-palmitoyl acyltransferases. We developed qRT-PCR probes that allow us to detect all of them effectively. From our studies, we have found that 5 DHHC genes are highly expressed in the NRAS mutant cell lines. By comparing the mass spec results and the mRNA profiling, we have narrowed down to 2 candidate genes which are highly enriched in NRAS mutant melanoma.



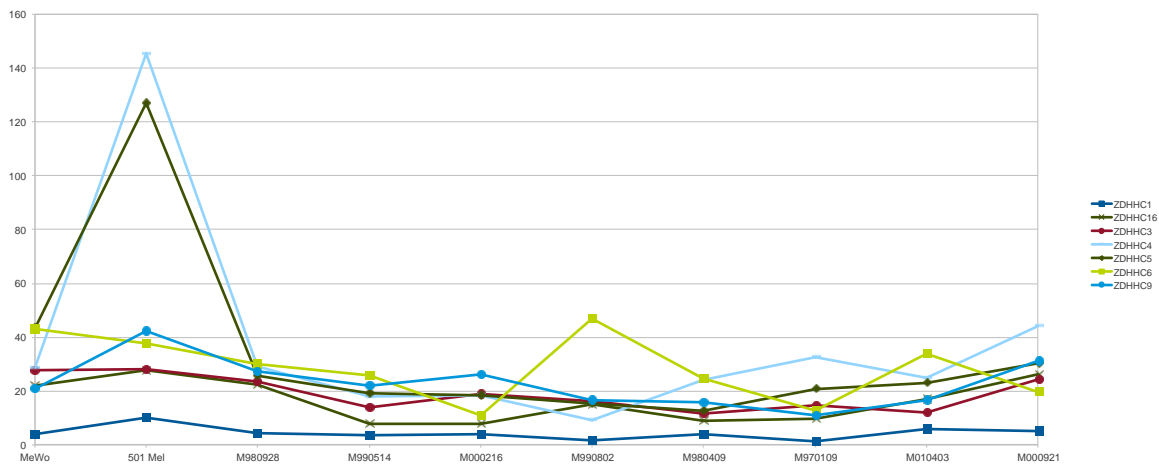
**Figure 1. Chemical Probe Labeling of NRas and BRaf mutant melanoma cells.**

NRas mutant melanoma cells (SK-Mel2 and SK-Mel30) and BRaf mutant melanoma cells (IPC-298, A375) are treated with alkyne-2-BP (50μM) for 8 h. The cell lysates reacted with biotin-azide with Cu catalyst (Click chemistry). The labelings of palmitoyl acyltransferase or palmitoylated proteins are detected by streptavidin blots. The red box

highlighted the protein bands uniquely labeled in NRas mutant melanoma cells.

**Task 1c.** Carry out bioinformatics studies of the expression profiles in a large set of melanoma cell lines from previously published datasets (month 3-6).

**Accomplishments:** We carried out bioinformatics studies by data mining of public available expression profiles. From NCBI GEO data bases, we have identified melanoma expression profiles. We found that DHHC4 and DHHC5 are among the highly expressed PATs in NRAS mutant cells (**Figure 2**). These results support some of our findings using qRT-PCR methods described above, but not necessarily be the only methods to provide candidate PATs.



**Figure 2. Bioinformatic analysis of melanoma samples using NCBI GEO dataset (GSE17349).** The studies included NRas (G12D) mutant cell line (501Mel) confirmed by sequencing, and other melanoma cell lines and patient-derived samples. The expression data were analyzed across all ZDHHC-family proteins. ZDHHC4 and ZDHHC5 are highly expressed in NRas mutant melanoma cell line, but not in other cell lines.

**Task 1d.** Histology studies of PATs in melanoma cells to confirm that the protein levels of PATs are elevated in N-RAS mutant melanoma cell (month 6-12).

**Accomplishments:** We have studied the staining of a commercially available antibody for one of the top candidate PATs. However, this antibody did not recognize the correct protein in Western blot or immunofluorescent staining. The antibody recognizes a different protein with a different size on the Western blot. The information showed on the vendor website is actually not correct. Therefore, there is no commercially available antibody could be used to study the protein level of the candidate PATs in histology. To address this issue, we will try to develop a new antibody that could recognize the protein. Alternatively, we will look at the expression level of the candidate PATs using data mining and bioinformatics methods as well as qRT-PCRs using primary tumor samples from a tissue array. This effort is on going and will likely to accomplished in the second year of the study.

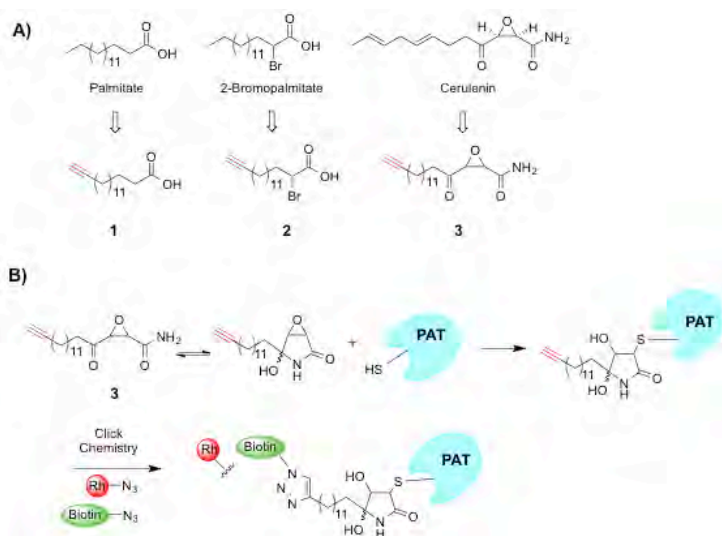
**Task2.** To evaluate the effects of shRNAs targeting DHHC-PATs on NRAS activity, melanoma cell proliferation and apoptosis (Month 13-24):

Task 2 will be carried out in the second year, and we will report our progress and findings in the next annual report.

**Task3.** To design and synthesize analogues of cerulenin, a natural product inhibitor of palmitoyl acyltransferases to identify small molecule inhibitors of N-RAS palmitoylation. (in parallel with Task1 and 2, Month 0-24):

**Task 3a.** Synthesize compound libraries based on the structure of cerulenin. Modifications of their head and tail groups might improve their selectivity and potency. ~50 compounds will be synthesized in 0-12 month.

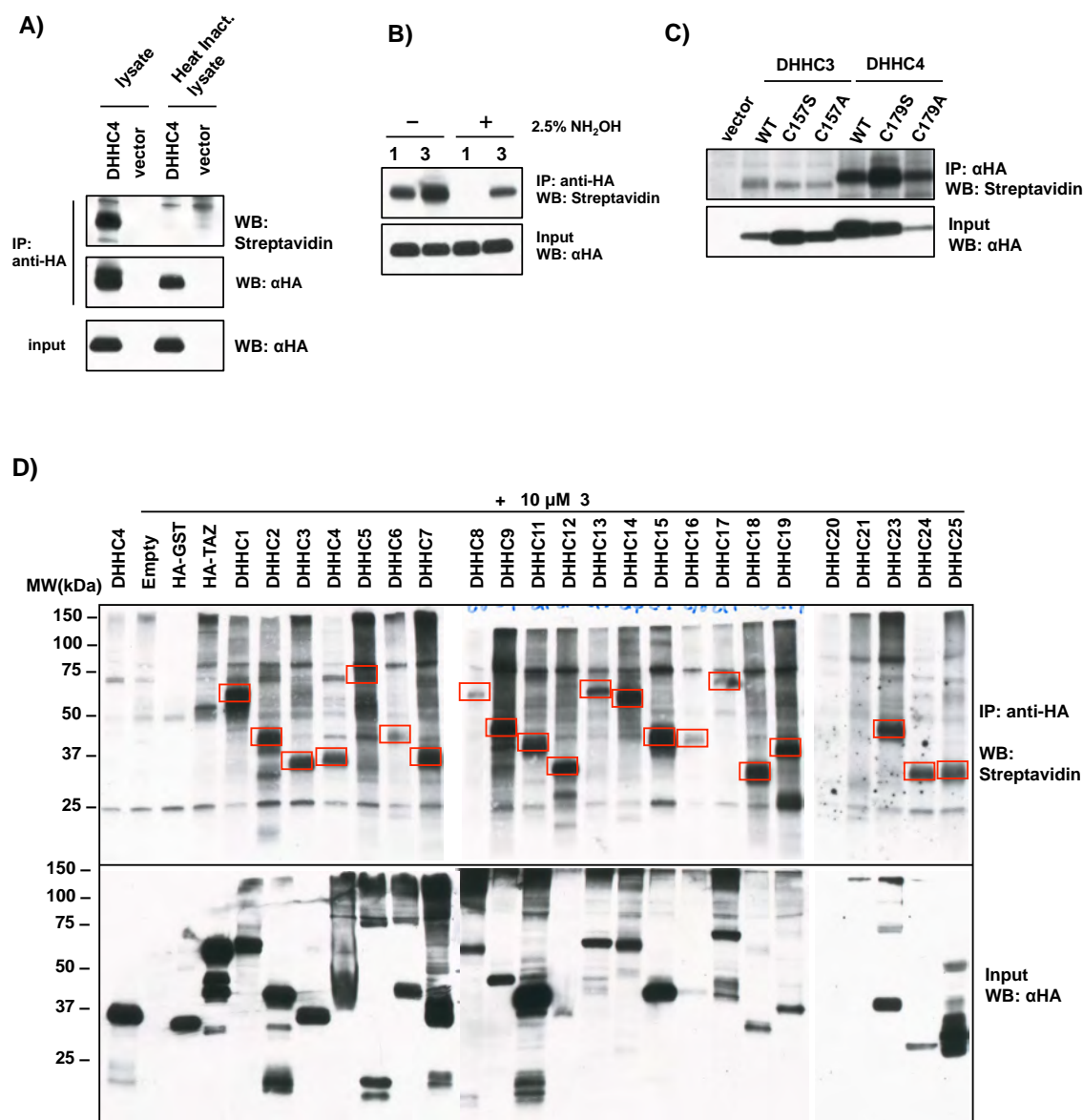
**Accomplishments:** We have designed cerulenin-based chemical probes to study palmitoyl acyltransferases. Cerulenin is a natural product inhibitor of fatty acid biosynthesis and protein palmitoylation. It has been shown that a palmitoyl analogue for cerulenin has good selectivity over fatty acid synthases. We have synthesized an alkyne analogue of cerulenin and a synthetic strategy has been developed (**Figure 3**).



**Figure 3. Design of Cerulenin-based chemical probes.** (A) structures of the substrate labeling probe **1**, PATs activity probe **2** based on 2-bromopalmitate and probe **3** based on cerulenin. (B) Labeling and detection of palmitoyl acyltransferases by probe **3**. Figure adapted from our publication: Zheng et al. ACS Chem. Biol. 2015.

We then tested this probe in cells express HA-DHHC4. We found that this probe effectively labels DHHC proteins in doses lower than 2-BP based first-generation probes. More over, this probe labels protein in vitro in cell lystate, suggesting that live cell is not required for the labeling. This new probe will provide additional chemical tools to study palmitoylation and palmitoyl acyltransferases in melanoma cells (**Figure 4**). It will serve as a base to develop high throughput assays to identify small molecule inhibitors of palmitoyl acyltransferases.

Using the synthetic route that we developed in this task, we have synthesized several analogues of cerulenin with variations at the head groups. We have finished synthesis of ~10 compounds. More analogues are planned and will be made in the second year of studies.



**Figure 4.** (A) 10 μM of probe 3 could label DHHC4 in vitro in cell lysates, but not in heat-inactivated lysate. (B) Probe 3 stably labeled DHHC4 and formed hydroxylamine-resistant adduct, whereas probe 1 forms a labile thioester intermediate with the enzyme. (C) Mutations of cysteine residue at the DHHC-domain in DHHC3 and 4 do not disrupt the labeling of probe 3. (D) Probe 3 labeled DHHC-family of palmitoyl acyltransferases. The red boxes indicated the labeled DHHC proteins based on their molecular weight and positions in the anti-HA Western blot.

**What opportunities for training and professional development have the project provided?**

The PI has strong background in chemistry and drug discovery. His career goal is to become a leading scientist and independent investigator in melanoma and skin cancer drug discovery. To achieve the career goal, the mentor (Dr. Fisher) has been guiding Dr. Wu's melanoma research through routine meetings and discussions. Dr. Wu has participated the MGH melanoma program weekly meeting chaired by Dr. Fisher. Dr. Wu has attended the melanoma workshops sponsored by Dana-Farber/Harvard Cancer Center (DF/HCC) and the Koch Institute of Cancer Research at MIT, which was organized by Dr. Fisher. These workshops covered melanoma clinical trials studies, melanoma genomics and drug discovery, and will provide additional training and collaboration opportunities for young investigators. Dr. Wu has participated the melanoma journal club and seminars in MGH and Harvard community, where he will learn the progresses in melanoma research.

**How were the results disseminated to communities of interest?**

Nothing to report.

**What do you plan to do during the next reporting period to accomplish the goals?**

As we planned in the original SOW, we will focus on Task 2 to fully validate the candidate PATs and their functions in melanoma. We will carry out shRNA knockdown experiments to show whether these PATs are essential for NRAS mutant melanoma proliferation and survival.

#### **4. IMPACT**

**What was the impact on the development of the principle discipline of the project?**

We have demonstrated that a chemical approach using Activity based protein profiling (ABPP) could study the palmitoyl acyltransferases in melanoma. We have developed multiple chemical tools to study PATs in cancer cells. Our study has identified candidate PATs possibly as new therapeutic targets, which could have significant impact in drug discovery and cancer research.

**What was the impact on other disciplines?**

Nothing to report

**What was the impact on technology transfer?**

Nothing to report

**What was the impact on society beyond science and technology?**

Nothing to report

## 5. CHNAGES/PROBLEMS

### Changes in approach and reasons for change:

As we stated in the Accomplishment section, Task 1d of histology studies of PATs in the melanoma cells requires changes. The commercial antibody for the DHHC proteins failed to recognize the correct protein, and could not be used in histology studies. We have changed to use qRT-PCR to evaluate the expression of the candidate PATs in melanoma samples, which will provide similar results.

## 6. PRODCUTS

**Publications:** A manuscript describing part of the work has been published on **ACS Chemical Biology**.

“A Clickable Analogue of Cerulenin as Chemical Probe to Explore Protein Palmitoylation” Baohui Zheng, Shunying Zhu, and Xu Wu\*, **ACS chemical biology**, 2015 Jan 16;10(1):115-21. doi: 10.1021/cb500758s. Epub 2014 Oct 23.

**Acknowledgement of federal support:** Yes

### Other products:

Research material: chemical probes for palmitoyl acyltransferases (16C-BYA and 16-EYA)

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

### What individuals have worked on the project?

Name:	Xu Wu
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-1624-0143
Nearest person month worked:	3
Contribution to project:	Dr. Wu has supervised the research, designed the experiments and interpreted the results
Funding support:	MGH Institutional fund

	American Cancer Society  Melanoma Research Alliance  National Cancer Institute
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Name:	Baohui Zheng
Project Role:	Research Fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6
Contribution to project:	Dr. Zheng has performed work in the area of synthesis, chemical labeling and mass spectrometry
Funding support:	Melanoma Research Alliance

Name:	Michael DeRan
Project Role:	Research Fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3
Contribution to project:	Dr. DeRan has performed work in the area of biochemistry, and mRNA expression profiling
Funding support:	MGH Institutional fund  American Cancer Society  National Cancer Institute

**Has there been a change in the active other support of the PD/PI(s)?**

A previously listed pending application has been awarded for the PI.

National Cancer Institute (NCI)/NIH, Wu (PI)

1R01CA187909-01

Metabolic regulation of cellular tight junction proteins

The goal of this proposal is to study the regulation of cellular junction proteins AMOTL1 by AMPK, and whether this regulation lead to inhibition of YAP oncogene in cancers in vitro and in vivo.

Overlap: None

This award will not impact the level of efforts for the DoD grant.

**What other organizations were involved as partners?**

Nothing to report.

## **8. SPECIAL REPORTING REQUIREMENT**

N/A

## **9. APPENDICES**

Yes.

PDF version of the publication.

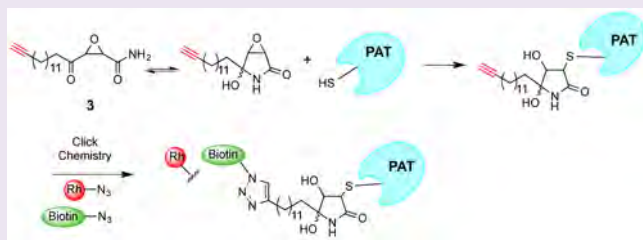
# Clickable Analogue of Cerulenin as Chemical Probe to Explore Protein Palmitoylation

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**S** Supporting Information

**ABSTRACT:** Dynamic palmitoylation is an important post-translational modification regulating protein localization, trafficking, and signaling activities. The Asp-His-His-Cys (DHHC) domain containing enzymes are evolutionarily conserved palmitoyl acyltransferases (PATs) mediating diverse protein S-palmitoylation. Cerulenin is a natural product inhibitor of fatty acid biosynthesis and protein palmitoylation, through irreversible alkylation of the cysteine residues in the enzymes. Here, we report the synthesis and characterization of a “clickable” and long alkyl chain analogue of cerulenin as a chemical probe to investigate its cellular targets and to label and profile PATs *in vitro* and in live cells. Our results showed that the probe could stably label the DHHC-family PATs and enable mass spectrometry studies of PATs and other target proteins in the cellular proteome. Such probe provides a new chemical tool to dissect the functions of palmitoylating enzymes in cell signaling and diseases and reveals new cellular targets of the natural product cerulenin.



Protein S-palmitoylation is the post-translational attachment of the 16-carbon palmitate to the cysteine residues of proteins through a thioester linkage.<sup>1,2</sup> In contrast to other lipid modifications of proteins, S-palmitoylation is a dynamic process. In response to extracellular or intracellular signals, the palmitoylation–depalmitoylation process precisely regulates the localization, trafficking, and cofactor binding properties of proteins.<sup>3</sup> A large number of palmitoylated proteins have been identified by biochemical and proteomic methods.<sup>4–7</sup> Palmitoylation is essential for the signaling functions of Src-family kinases, Ras GTPase, G-proteins, G-protein coupled receptors (GPCRs), and synaptic adhesion molecules.<sup>8</sup> For example, palmitoylation of the two C-terminal cysteine residues is required for the proper trafficking and membrane localization of H-Ras and N-Ras.<sup>9</sup> Disrupting the palmitoylation and depalmitoylation cycle could effectively block their oncogenic functions, suggesting that targeting protein palmitoylation could provide potential therapeutic options for diseases.<sup>10</sup> Therefore, it is important to develop chemical tools to dissect the functions of palmitoylation in signaling and diseases.

Protein S-palmitoylation can be mediated by nonenzymatic or enzymatic processes.<sup>1</sup> Some proteins bind to palmitoyl-Coenzyme A (CoA) directly, resulting in thioester exchange reactions with the proximal cysteine residues in the proteins.<sup>11</sup> For example, purified G $\alpha$  protein could be autopalmitoylated when incubated with palmitoyl-CoA.<sup>12</sup> Therefore, the concentration of cellular palmitoyl-CoA pool could greatly influence the level of autopalmitoylation. Yeast genetic studies have revealed that enzymatic palmitoylation involves a family of highly conserved protein palmitoyl acyltransferases (PATs).<sup>13,14</sup> These enzymes are generally transmembrane

proteins with a conserved Asp-His-His-Cys (DHHC) domain in the active site. The human genome encodes 23 DHHC-family PATs. These enzymes are usually localized in the ER, Golgi, or at the plasma membrane of cells and regulate the palmitoylation of diverse protein substrates. Genetic and biochemical studies have provided insights to the functions of many DHHC enzymes and their substrates.<sup>15</sup> For example, mice with a loss-of-function mutation of DHHC13 showed multiple aging related phenotypes, including alopecia, osteoporosis, systemic amyloidosis, and early death.<sup>16</sup> The *dhhc17*<sup>−/−</sup> mice display neurodegenerative phenotypes, highlighting the function of DHHC17 in regulation of synaptic and neuronal functions.<sup>17</sup> A point mutation in DHHC21 was identified in the *depilated* (*dep*) mouse mutant, resulting in hair follicle degeneration.<sup>18</sup> Furthermore, DHHC9, 11, 14, and 17 proteins are shown to be deregulated in bladder, lung, and colon cancers and in leukemia and could contribute to oncogenic processes.<sup>19–21</sup> In addition to the S-palmitoylation, membrane-bound O-acyltransferases (MBOAT) such as Porcupine and Hedgehog acyltransferase (Hhat) could mediate the O- and N-palmitoylation of Wnt and Hedgehog proteins, respectively.<sup>22,23</sup> Interestingly, acyltransferases, which were previously unrecognized as PATs, might catalyze protein palmitoylation. For example, lysophosphatidylcholine acyltransferase 1 (Lpcat1) has been reported to mediate the O-

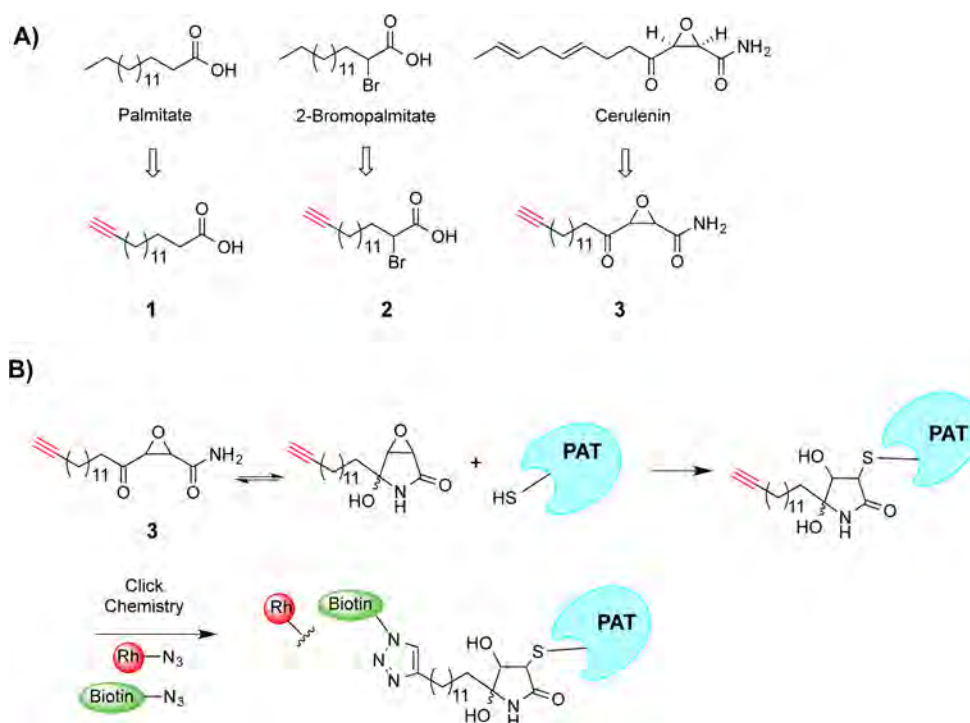
**Special Issue:** Post-Translational Modifications

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**Figure 1.** Chemical probes to study protein palmitoylation. (A) Structures of the substrate labeling probe 1, PATs activity probe 2 based on 2-bromopalmitate, and probe 3 based on cerulenin. (B) Labeling and detection of palmitoyl acyltransferases by probe 3.

palmitoylation of histone H4 protein.<sup>24</sup> These reports highlight the importance of identifying new palmitoylating enzymes or autoacylating proteins and revealing their functions in signaling and diseases.

To profile and study palmitoylation, analogues of palmitate, such as 15-hexadecynoic acid (1), have been widely used as chemical probes to metabolically label palmitoylated proteins (substrates).<sup>25</sup> We sought to develop chemical probes to covalently label and profile PATs (enzymes) to elucidate their functions in signal transduction. Previously, we have reported the activity-based protein profiling (ABPP) probe (2) for PATs, based on the irreversible and pan-inhibitor 2-bromopalmitate (2-BP) (Figure 1A).<sup>26</sup> Probe 2 (20–100  $\mu$ M) could effectively label PATs in live cells, but not in cell lysates *in vitro*, suggesting that metabolic conversion to its CoA derivatives might significantly enhance its activity. We used probe 2 to label and profile endogenous palmitoyl acyltransferases in cells and have identified many acyltransferases and palmitoylated proteins, suggesting that 2 could also be incorporated into the cellular lipid pool and utilized as an acyl donor. To improve the efficiency and specificity of the labeling, we sought to synthesize and characterize new chemical probes for PATs and autopalmitylated proteins.

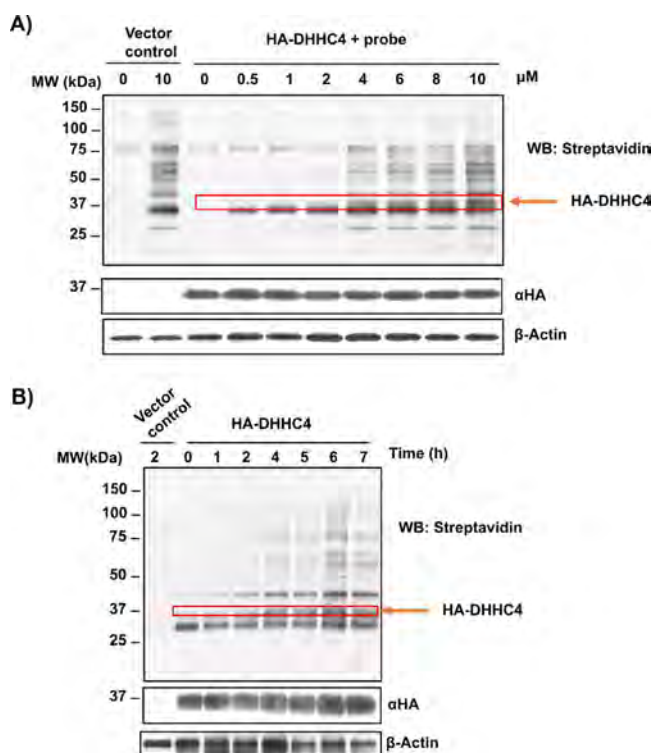
The natural product cerulenin ([2R,3S]-2,3-epoxy-4-oxo-7,10-*trans,trans*-dodecadienoylamide) has been studied as an antifungal and antibacterial agent for nearly 30 years and known to inhibit fatty acid synthesis and protein palmitoylation.<sup>27</sup> The structure of cerulenin is composed of an  $\alpha$ -keto-epoxycarboxamide with an octadienyl side chain. It exists in an equilibrium between the open chain and a cyclized hydroxylactam form. The epoxycarboxamide moiety has been proposed as the “warhead” of the compound, which alkylates the cysteine residues in fatty acid synthase and palmitoyl acyltransferases, leading to irreversible inhibition.<sup>28</sup> We recently found that cerulenin can compete with probe 2 in labeling of DHHC

domain containing PATs, suggesting that it might occupy the same or an adjacent binding site of acyl-CoA and inhibit the acyl-enzyme adduct formation.<sup>26</sup> Previously, the structure–activity relationship (SAR) studies of  $\sim$ 30 cerulenin analogues have shown that the  $\alpha$ -keto-epoxide moiety is essential for the palmitoylation inhibition activity.<sup>29</sup> Interestingly, analogues with a long saturated aliphatic chain (15–17 carbon chain) showed improved potency in inhibiting H-Ras and N-Ras palmitoylation, while not inhibiting fatty acid synthesis.<sup>29</sup> On the basis of these reports, we hypothesized that a “clickable” long aliphatic chain analogue of cerulenin might serve as a chemical probe to directly label palmitoylating enzymes, allowing proteomic and imaging studies of palmitoyl acyltransferases *in vitro* and in cells (Figure 1B).

Toward this end, we synthesized the cerulenin analogue 3 (cis-2,3-epoxy-4-oxooctadec-17-ynamide) as a chemical probe for palmitoylating enzymes (Figure 1B). The synthesis of 3 was achieved as illustrated in Scheme S1 in Supporting Information, modified from previously reported methods.<sup>30</sup> Notably, the key intermediate  $\gamma$ -lactone was synthesized through selenolactonization to introduce the terminal alkyne. Specifically, (*E*)-octadec-3-en-17-ynoic acid was added to PhSeCl solution with Et<sub>3</sub>N in CH<sub>2</sub>Cl<sub>2</sub> at  $-78$  °C. The mixture was warmed to  $-40$  °C over 40 min to afford the intermediate, which was then oxidized by 35% of H<sub>2</sub>O<sub>2</sub> for 40 min. The desired product octadec-2-en-17-ynoic acid  $\gamma$ -lactone can be synthesized with good yield (50%). The final product 3 can be readily made with 15% of overall yield. The detailed synthetic procedures and compound characterization are described in the Supporting Information.

We set out to test whether probe 3 could label exogenous PATs in cells first. We transfected HEK293 cells with expression vectors carrying hemagglutinin (HA)-tagged mouse DHHC4. Forty-eight hours after transfections, we treated the cells with various concentrations of probe 3 (0.5 to

10  $\mu\text{M}$ ) and incubated for 5 h. We isolate the cell lysates and subsequently carried out Cu-mediated 1,3-dipolar cycloaddition (Click reaction) with biotin-azide. The labeling efficiency was evaluated by streptavidin blot. As shown in Figure 2A, we could



**Figure 2.** Cerulenin analogue 3 labeled HA-tagged DHHC4 protein in HEK293 cells. (A) HEK293 cells transfected with vector control or HA-DHHC4 expression vector were treated with different concentrations of probe 3 for 5 h. The labeling of DHHC4 was detected by Click chemistry using biotin azide followed by streptavidin blot. The expression of DHHC4 was confirmed by Western blot using anti-HA antibody. (B) HEK293 cell transfected with vector control or HA-DHHC4 were treated with 10  $\mu\text{M}$  of probe 3 for the indicated time.

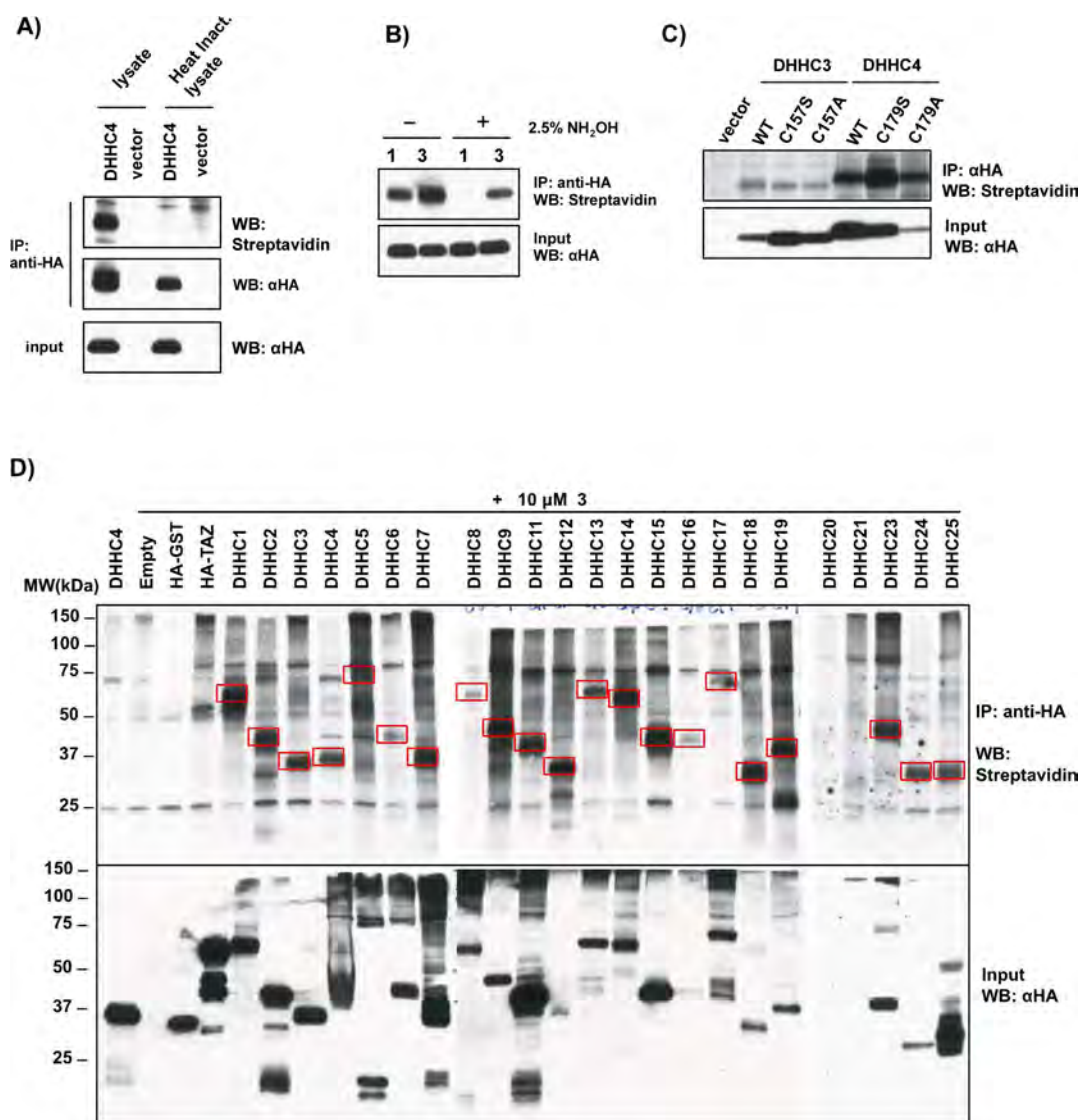
detect the labeling of DHHC4 by probe 3 at concentrations as low as 2  $\mu\text{M}$ , consistent with reported cellular activity of cerulenin in inhibition of palmitoylation. Probe 3 appeared to be more potent than previously reported 2-BP-based probe 2, which effectively labeled DHHC4 proteins at concentrations around 50–100  $\mu\text{M}$ . The improved potency of labeling might be resulted from the stronger chemical reactivity of the epoxide warhead in the probe. We then tested the time dependency of the labeling. Similarly, we transfected cells with HA-DHHC4 expression vector, and then treated cells with 3 at 10  $\mu\text{M}$ . We isolated the cell lysate at various time points (1 to 7 h), followed up with Click chemistry with biotin-azide and Western blotting. We observed that probe 3 could label exogenous DHHC4 after 1 h of incubation. The labeling efficiency increased with longer incubation time and peaked after 4–6 h of incubation (Figure 2B). Overall, probe 3 has better efficiency and potency to label DHHC4 protein in cells, compared to our previously reported probe 2.

It is known that 2-BP and probe 2 could be metabolically converted to the Coenzyme A (CoA) derivatives in live cells, and the CoA derivatives are much more potent and effective to alkylate DHHC4. Such properties might limit the *in vitro* application of probe 2, such as in enzyme-based high

throughput assays, where purified enzymes in cell-free systems are preferred. In contrast, cerulenin might directly alkylate the protein by epoxide ring opening reactions *in vitro*, thus metabolic conversion in live cells are not required for its labeling activity. We isolated cell lysates from HEK293 cells expressing HA-DHHC4 using nondenaturing conditions and tested whether probe 3 could directly label PATs *in vitro* in cell lysates. We treated cell lysate or heat-inactivated lysate with probe 3 for 5 h. We immunoprecipitated HA-tagged DHHC4 with anti-HA antibody and then proceeded to perform the Click reaction with biotin-azide. The active DHHC4 enzyme in the lysate could be labeled, while heat-inactivated DHHC4 could not, suggesting that 3 could label native DHHC4 protein, but not the denatured enzyme *in vitro* (Figure 3A). In addition, the labeling required PATs in their native, nondenatured state, suggesting the labeling might not be a result of nonspecific alkylating of cysteine residues in the proteins, but rather through binding and alkylating specific residues around the binding site.

15-Hexadecynoic acid (1), the 16-carbon fatty acid with terminal alkyne group, has been widely used as a reporter of palmitoylated proteins.<sup>25</sup> It mimics palmitate and forms a labile thioester linkage with the substrate proteins, which can be removed by treatment with 2.5% hydroxylamine ( $\text{NH}_2\text{OH}$ ). It labels DHHC-family PATs by forming an acyl-enzyme intermediate or labels the peripheral palmitoylation sites of PATs.<sup>31</sup> However, treatment of hydroxylamine readily removed the thioester adduct formed by 1 and DHHC4. As cerulenin alkylates DHHC proteins by epoxide ring opening, it should form a stable adduct with the enzyme. We treated HEK293 cells expressing HA-DHHC4 with probes 1 and 3 for 5 h, and the cell lysates were subsequently treated with or without 2.5% hydroxylamine for 5 min. We evaluated the probe labeling by Click chemistry with biotin-azide and streptavidin blot. Indeed, cerulenin-based probe 3 forms a hydroxylamine-resistant stable adduct with DHHC4 protein, where the control probe 1 forms labile adduct with the enzyme (Figure 3B). This result is consistent with the proposed mechanism that probe 3 could directly alkylate the enzyme.

The DHHC family of PATs contain a cysteine-rich domain including the Asp-His-His-Cys (DHHC) catalytic motif. Mutations of the cysteine in the DHHC motif to serine or alanine residues abolished the acyl transferase activity and prevented enzyme autoacylation.<sup>32</sup> Previously, we have shown that the 2-BP-based probe 2 labels the wild-type enzyme but not the catalytically inactive mutant enzymes, suggesting that 2 is an activity-based probe for PATs and the conserved cysteine residue in the DHHC motif might be the site of labeling.<sup>26</sup> To test whether the cerulenin analogue 3 has similar labeling activity, we transfected cells with HA-tagged wild-type DHHC3 and 4 or their mutants (DHHC3, C157S and C157A; DHHC4, C179S and C179A, respectively), where the conserved cysteine residues in the DHHC motif were mutated to serine or alanine residues. Interestingly, 10  $\mu\text{M}$  3 effectively labeled not only the wild-type proteins but also the mutants. These results strongly suggest that cerulenin or its analogue 3 alkylates other cysteine residues proximal to its binding site (Figure 3C), and the core cysteine residue at the DHHC motif might not be the sites of alkylating. Moreover, as the enzymatically inactive proteins can still be labeled, probe 3 would not be characterized as an activity-based probe, but rather a reactive chemical probe that could bind to and alkylate PATs. These results also confirmed previous speculation that cerulenin and probe 3 are not a



**Figure 3.** (A) Ten micromolar probe 3 could label DHHC4 *in vitro* in cell lysates, but not in heat-inactivated lysate. (B) Probe 3 stably labeled DHHC4 and formed hydroxylamine-resistant adduct, whereas probe 1 forms a labile thioester intermediate with the enzyme. (C) Mutations of cysteine residue at the DHHC domain in DHHC3 and 4 do not disrupt the labeling of probe 3. (D) Probe 3 labeled DHHC family of palmitoyl acyltransferases. The red boxes indicated the labeled DHHC proteins based on their molecular weight and positions in the anti-HA Western blot.

mechanism-based irreversible inhibitor of PATs and mechanistically different from 2-BP and probe 2, offering different labeling tools for PATs. Several disease relevant mutations in DHHC proteins might disrupt their catalytic activities, such as the loss-of-function mutation of DHHC13 in the systemic amyloidosis mouse model. Such mutant PATs could be labeled by probe 3 but not probe 2. Profiling the samples with these two probes in parallel could provide an approach to study the abundance of catalytically defected vs catalytically active PATs in these disease-relevant models.

As described previously, although 2-BP based probes could label most DHHC-PATs tested, they failed to label several important PATs, including DHHC1, 8, and 23.<sup>26,33</sup> To evaluate whether cerulenin-based probe 3 could have better generality and versatility in labeling members of DHHC-family PATs, we expressed all 23 HA-tagged DHHCs in HEK293 cells and carried out labeling experiments with 10  $\mu\text{M}$  3 for 5 h. We immunoprecipitated the DHHC proteins using anti-HA antibodies and carried out the Click reaction with biotin-

azide. The streptavidin blot in Figure 3D shows that all of the expressed PATs could be successfully labeled with 3. DHHC20 and DHHC21 were poorly expressed in HEK293 cells and have relatively weak labeling. We observed that the intensity of the labeling largely correlates with the expression levels of the proteins in the lysates. However, probe 3 has higher labeling efficiency for DHHC12 and DHHC18 but weaker efficiency for DHHC6 and DHHC8. Such differences in labeling activity might be resulted from the differences in binding affinity of the probe toward different DHHC-PATs or the reactivity of the proximal cysteine residues in the proteins. Nevertheless, DHHC1, 8, and 23 could be successfully labeled by 3, suggesting that probe 3 offers broader coverage of the DHHC family of PATs compared with previously reported probes. Taken together, we have shown that the probe 3 could be used as a chemical probe to label and profile the DHHC-family PATs and is superior to previously reported probes with broader labeling activity and higher potency.

**Table 1. Representative Proteins Labeled by Probe 3 and Could Be Competed off by 2-BP and Cerulenin<sup>a</sup>**

symbol	protein name	DMSO	probe 3	probe 3 + 2BP	probe 3 + cerulenin
RTN4	reticulon-4	4	164	74	78
VDAC2	voltage-dependent anion-selective channel protein 2	11	140	30	40
FABP5	fatty acid-binding protein, epidermal	4	103	1	9
VDAC3	voltage-dependent anion-selective channel protein 3	3	101	48	49
HMOX2	heme oxygenase	1	100	43	45
ATP2A2	sarcoplasmic/endoplasmic reticulum calcium ATPase 2	6	94	30	54
CYB5B	cytochrome b5 type B	3	63	17	18
ATP2A1	sarcoplasmic/endoplasmic reticulum calcium ATPase 1	5	61	29	38
HM13	minor histocompatibility antigen H13	0	55	21	16
SEC63	translocation protein SEC63 homologue	0	38	4	5
TXNDC5	thioredoxin domain-containing protein 5	6	38	20	11
TRAPPC3	trafficking protein particle complex subunit 3	0	36	2	5
ENDOD1	endonuclease domain-containing 1 protein	0	35	5	6
MTAP	S-methyl-5'-thioadenosine phosphorylase	0	34	11	16
HCCS	cytochrome c-type heme lyase	0	33	8	7
APOL2	apolipoprotein L2	0	32	7	9
TMX1	thioredoxin-related transmembrane protein 1	0	31	18	17
MLANA	melanoma antigen recognized by T-cells 1	3	28	4	7
BACH1	transcription regulator protein BACH1	0	27	2	4
CD81	CD81 antigen	4	25	5	5
LPCAT2	lysophosphatidylcholine acyltransferase 2	0	25	2	4
COMT	catechol O-methyltransferase	2	24	16	8
HEATR3	HEAT repeat-containing protein 3	0	21	6	1
ZDHHC20	probable palmitoyltransferase ZDHHC20	0	19	4	7
AP1AR	AP-1 complex-associated regulatory protein	0	16	2	0
GLTP	glycolipid transfer protein	0	13	1	0
ALG6	dolichyl pyrophosphate Man9GlcNAc2 alpha-1,3-glucosyltransferase	0	12	1	0
MIEN1	migration and invasion enhancer 1	0	12	0	0
UBR7	Putative E3 ubiquitin-protein ligase UBR7	0	11	0	0
LPCAT1	Lysophosphatidylcholine acyltransferase 1	0	9	1	2
ST6GALNAC3	alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase 3	0	9	0	0
RABGGTB	geranylgeranyl transferase type-2 subunit beta	0	8	0	1
C18orf32	UPF0729 protein C18orf32	0	7	0	1
VKORC1	vitamin K epoxide reductase complex subunit 1	0	5	0	0
EPT1	Ethanolaminephosphotransferase 1	0	4	0	0

<sup>a</sup>The number of total matched peptide spectra of representative acyltransferases, PATs, and other abundant proteins is listed. The full list of identified proteins can be found in the Supporting Information.

To identify the cellular targets of the cerulenin analogue **3**, we carried out labeling experiments in 501Mel melanoma cells with 10  $\mu$ M **3** for 5 h. The cell lysates were then subjected to Click chemistry using biotin-azide and enriched by streptavidin beads. The labeled proteins were digested on beads by trypsin and analyzed by mass spectrometry. We filtered the mass spectrometry results for proteins with >2 spectra counts in duplicate and with >5-fold enrichment over the DMSO control samples. We have identified >200 total proteins as putative cellular targets of probe **3**, suggesting that the cerulenin analogue could alkylate diverse proteins in cells (Table 1 and Supplementary Table 1). Among them, we have identified endogenous palmitoyl acyltransferase ZDHHC20. ZDHHC5, ZDHHC4, and ZDHHC6 were also identified by mass spectrometry from these cells but did not pass the stringent threshold. Many other acyltransferases and acyl-CoA binding enzymes were also labeled by **3**, including lysophosphatidylcholine acyltransferase 1 (LPCAT1), which is reported as O-palmitoylation enzyme for histone H4; and MBOAT7, a member of membrane-bound acyltransferases. The most abundant targets enriched by probe **3** include reticulon 4 (RTN4), heme oxygenase 2 (HMOX2), voltage-dependent

anion channels (VDAC2 and VDAC3), and ER calcium ATPase (ATP2A2). Interestingly, these proteins are also targets of the 2-BP based probes, suggesting that these two probes have some overlapping cellular targets. However, several proteins, which are labeled by 2-BP probes, including carnitine O-palmitoylacyltransferase (CPT1), were not among the proteins identified by probe **3**, suggesting that these probes have different labeling specificity toward certain acyltransferases. Several common targets, such as reticulon, cytochrome b5, FLOT2, and ESYT2 are also known as palmitoylated proteins (substrates). As cerulenin and 2-BP could inhibit PATs activities in cells, it is interesting to further study whether these common targets were palmitoylated through nonenzymatic processes or palmitoylated by PATs, which were not inhibited by 2-BP and cerulenin. More importantly, we observed several proteins, which are uniquely labeled by probe **3**. For example, probe **3** could label acyl protein thioesterase 1 (APT1/LYPLA1), which is the enzyme responsible for depalmitoylation. Furthermore, we did not identify fatty acid synthase complex proteins from the mass spectrometry studies, consistent with the notion that the long alkyl chain analogue

of cerulenin specifically inhibits palmitoylation but not fatty acid synthase.

To further reveal whether the protein targets labeled by probe 3 are common targets of cerulenin and 2-BP involved in palmitoylation, or cerulenin or probe 3 specific targets, we carry out competition labeling and mass spectrometry studies of probe 3. We pretreated 501Mel cells with 2-BP (100  $\mu$ M) or cerulenin (10  $\mu$ M) for 4 h, and then added probe 3 (5  $\mu$ M) to the cells. We isolated the cell lysates after 4 h of probe incubation and carried out Click chemistry with biotin-azide as described previously. We observed that certain bands are indeed competed off by 2-BP and cerulenin (Supplementary Figure S1). To reveal the identities of common target proteins of 2-BP, cerulenin and probe 3, and protein targets that are specific for cerulenin, we enriched the labeled proteome using streptavidin beads and then carried out on-beads digestion by trypsin and then analyzed the proteins by mass spectrometry. We observed that ~140 proteins, which can be competed off by 2-BP and cerulenin (with >2-fold less total peptide spectra counts in the presence of competitors), suggesting that these proteins are common targets of these two palmitoylation inhibitors and probe 3. These common targets include the known palmitoylated protein or PATs, such as reticulon 4, VDAC2, ZDHHC20, and LPCAT1. Interestingly, we also observed that ~15 proteins can be competed off by cerulenin, but not 2-BP. These proteins, including thioredoxin reductase 1 (TXNRD1), exportin 1 (XPO1), and GPR143, might represent protein target specific for cerulenin and probe 3, which should have different reactivity and specificity than 2-BP and might label proteins not involved in palmitoylation. Furthermore, we have observed several proteins that can be competed off by 2-BP, but not cerulenin, including cholesterol ester hydrolase 1 (NCEH1), PML protein, and acyl protein thioesterase (LYPLA1/APT1). As the long alkyl chain moiety in probe 3 could improve its specificity toward palmitoylating proteins compared to cerulenin, these targets might represent palmitoylated proteins that cerulenin could not label. In summary, we could evaluate whether the proteins are cerulenin-specific targets or palmitoylation-related targets by competition assays.

To assess whether our methods could identify novel palmitoylated/palmitoylating proteins, we chose to validate whether lysophosphatidylcholine acyltransferase 2 (LPCAT2) and acyl protein thioesterase (LYPLA1/APT1) are indeed palmitoylated. We labeled the proteome of 501Mel cells for 8 h using the 15-hexadecynoic acid (1), which has been widely used as a reporter of palmitoylated proteins.<sup>25</sup> We then carried out Click chemistry using biotin-azide and immunoprecipitated the palmitoylated proteins using streptavidin beads. The bounded proteins were eluted using biotin-containing buffer. We then used anti-LPCAT2 or anti-LYPLA1 antibodies in Western blot. We have successfully detected that endogenous LPCAT2 and LYPLA1/APT1 are indeed palmitoylated (Supplementary Figure S2). A recent report suggested that APT1 palmitoylation might be involved in its localization at the Golgi apparatus.<sup>34</sup> Therefore, palmitoylation of APT1 could play important roles in regulating its biological functions. Further studies are needed to elucidate the detailed functions of palmitoylation in regulation of LPCAT2 and APT1.

Taken together, here we report synthesis and characterization of a novel chemical probe for protein palmitoylation and palmitoyl acyltransferases. Compared to previously reported PATs probes, probe 3 has several advantages. It has an

improved potency and could be used at much lower concentration *in vitro* and in cell culture. In addition, probe 3 does not require metabolic conversion in live cells and thus can directly label PATs *in vitro*. This feature allows its applications in enzymatic assays or developing cell-free assays for high throughput screening. More importantly, it labeled all the DHHC-PAT family members we have tested, suggesting it is a much broader and more universal chemical probe to profile PATs. We also successfully enriched the cellular targets of this probe and identified endogenous PATs and thioesterase from the proteome. Therefore, it could be used as a chemical probe to facilitate the identification of additional palmitoylating enzymes and autoacylated proteins involved in signaling and diseases. From our mass spectrometry studies, we found that probe 3 could also label proteins that are not previously known to be involved in palmitoylation. These proteins could be the new cellular targets of cerulenin. This is not surprising for irreversible chemical probes, which often target multiple proteins of different classes. For example, an alkyne-aspirin probe has been shown to label more than 120 proteins, and a majority of them were not previously identified as targets of aspirin.<sup>35</sup> Nevertheless, such covalent probes significantly expand our knowledge of new cellular targets of known drugs and natural products, and such polypharmacological effects might be the underlying mode-of-action of these compounds. Previously, epoxide containing oxirane probes were also reported to explore antibiotic targets in bacteria.<sup>36</sup> The long alkyl chain of cerulenin is a unique structural feature, which improves its specificity toward lipid binding and palmitoylating proteins in mammalian cells. This feature distinguishes it from a generic "epoxide" chemical probe, which might nonspecifically alkylate many cellular proteins. In summary, the clickable analogue of cerulenin represents a new chemical probe to explore palmitoylation and PATs in signal transduction. Further investigation of the functions of the identified target proteins could reveal new insights to this important posttranslational modification.

## METHODS

Material, synthetic procedures, and labeling methods are described in the Supporting Information.

## ASSOCIATED CONTENT

### Supporting Information

Synthesis, experimental protocols, full list of identified proteins from mass spectrometry studies, and supplementary figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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